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In Vitro Culture With Prednisolone Increases BCL-2 Protein Expression in Adult Acute Lymphoblastic Leukemia Cells

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The presence of *BCL-2* gene rearrangement has been detected also in cellular populations lacking the t(14;18) chromosomal translocation, such as B-lineage acute lymphoblastic leukemia (ALL) cells. It has been reported that overexpression of BCL-2 is related to resistance to glucocorticoid-induced apoptosis.

In this study, we aimed to evaluate whether in vitro culture with prednisolone (PDN) could modify the expression of BCL-2 protein. ALL cells from 21 patients were incubated for 72 hr with or without a minimally lethal (IC12) dose of PDN. In vitro culture with PDN did not affect the percentage of positive cells, even though the mean fluorescence index was significantly increased ($P = 0.0001$), thus indicating a higher level of protein production. These data could suggest a possible mechanism of drug resistance after treatment with PDN. © 1996 Wiley-Liss, Inc.

Key words: prednisolone, BCL-2, acute lymphoblastic leukemia

INTRODUCTION

BCL-2 gene rearrangement was initially found in non-Hodgkin's lymphomas showing the t(14;18) (q32;q31) chromosomal translocation [1]. The product of *BCL-2* gene is an inner mitochondrial membrane protein that has been described to inhibit programmed cell death (apoptosis) [2,3]. Recently, several investigators have reported the presence of high-level BCL-2 protein, even in cellular populations lacking both karyotypic and molecular evidence of chromosomal translocation, including normal lymphoid tissue [4], where high amount of protein appears to be produced in follicular mantle, composed of long-lived recirculating B cells. In acute myeloid leukemia (AML) cells, overexpression of BCL-2 protein is associated with prolonged survival in vitro in the absence of growth factors; patients show a lower complete remission rate after intensive chemotherapy and, ultimately, have a poor prognosis [5]. Also in lymphoid malignancies, an increased production of BCL-2 confers to cells the capacity of surviving in vitro [6] and, as demonstrated by gene transfer studies [7,8], a relative resistance to various chemotherapeutic agents. In vivo, BCL-2 does not seem to influence the outcome of acute lymphoblastic leukemia (ALL) patients [9], even though these data refer mainly to pediatric patients. In vitro, however, it has been reported that overexpression of BCL-2 protein renders pre-B-ALL cells more resistant to glucocorticoid-induced cell killing [10]. At present, however, few studies have been reported concerning the possibility of modulating BCL-2 by chemotherapeutic agents [11]. The aim of the present study was to evaluate whether in vitro culture in the presence of prednisolone (PDN) could modify the amount of BCL-2 protein production in adult ALL cells.

MATERIALS AND METHODS

ALL Cells

Heparinized bone marrow from 21 adult patients with newly diagnosed B-lineage ALL was obtained after informed consent. Mononuclear cells were collected after sedimentation on Ficoll-Hypaque (Lymphoprep, Nycomed Pharma, Oslo, Norway), adherent cells were eliminated after 1 hr adherence on plastic flasks. Nonadherent cells were resuspended in Iscove's modified Dulbecco's

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medium (IMDM, Gibco Europe, Paisley, UK), supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco).

Evaluation of Cytotoxicity

In this study, 5×10^6 ALL cells were cultured in 5 ml IMDM with 10% FCS. Prednisolone (Sigma Chemicals, St. Louis, MO, USA) was added at concentrations ranging from 100 ng/ml to 2,500 μg/ml. The choice of this drug concentration was based upon previously reported papers [12,13]; however, we increased the range by 1 log in order to have a better estimation of drug sensitivity. Control cultures received the same amount of media without the drug. After 72 hr of incubation at 37°C, the cells were counted electronically (Coulter Counter, model ZM, Coulter Electronics, Hialeah, FL, USA). Cell viability was also evaluated by trypan blue dye exclusion. Growth inhibition was calculated as a percentage of control culture cell number. Each experiment was performed in triplicate.

Immunofluorescence Evaluation of BCL-2

Immunodetection of intracellular BCL-2 protein was determined as previously reported [14]. Briefly, 2×10^6 cells (at baseline and after 72-hr incubation with PDN 1 μg/ml or with media alone) were fixed with paraformaldehyde/triton in phosphate-buffered saline (PBS). Before labeling, cells were incubated for 10′ with 2% heat-inactivated human AB serum, in order to prevent nonspecific binding of monoclonal antibody (MAb) to Fc receptors. Indirect immunofluorescence staining was then carried out using an anti BCL-2 MAb (Dako Italy SpA, Milan) and the isotype-specific fluorescin conjugated goat-anti-mouse antibody (Dako). Cells were then washed out and analyzed, using a FACScan flow cytometer equipped with Lysis II software (Becton Dickinson, San Jose, CA, USA). The results were expressed as percentage of positive cells (compared to background fluorescence, for which mouse IgG was used in place of MAb) and mean fluorescence intensity (MFI = sample mean channel : control mean channel ratio).

Statistical Analysis

Results were analyzed by the Wilcoxon test for paired data. Statistical significance was defined as $P < 0.05$ (two-tailed test).

RESULTS

Table I reports the clinical, immunological, and karyotypic characteristics of 21 ALL patients who entered the study. All the patients have been treated according to GIMEMA ALL 0288 protocol [15]. The dose-response curve concerning PDN-induced in vitro cytotoxicity on B-lineage ALL cells is reported in Figure 1. Under our

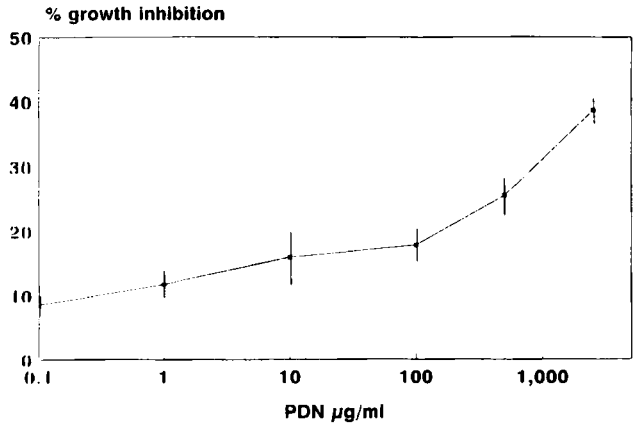

Fig. 1. Dose-response curve of PDN-induced in vitro growth inhibition of ALL cells. Data are expressed as mean ±SD of 21 cases.

experimental conditions, the mean IC50 of PDN after 72-hr incubation was higher than 2,500 μg/ml. Detection of BCL-2 was performed after culturing the cells with PDN 1 μg/ml; this drug concentration was chosen, as it was only minimally lethal (mean IC12 in the present set of experiments) on B-ALL cells.

A high percentage of BCL-2-positive cells was detected in all the samples at baseline (from 71% to 99%), these results are in line with those presented by other groups [6,14], showing a high level of BCL-2 protein in B-lineage leukemic cells. In vitro incubation with medium alone did not alter this pattern and, all the same, after treatment with PDN, no difference was observed in the percentage of positive cells (Table II). Mean fluorescence index (MFI) did not show any variation after incubation with medium alone, however, a significant increase was observed after incubation with PDN, as shown with the Wilcoxon test for paired data ($P = 0.0001$) (Fig. 2). Patients have been divided into three equal groups according to a different MFI level (group 1, less than 10% increase; group 2, more than 10% and less than 25% increase; group 3, more than 25% increase, compared to samples incubated without PDN). As shown in Table I, the three groups of patients did not display a different outcome of induction therapy, disease-free survival, and overall survival, presumably due to the low number of cases studied so far.

DISCUSSION

PDN-containing therapeutic regimens have widespread clinical use in ALL. Several studies in pediatric [12] and adult ALL [16] have demonstrated a shorter disease-free survival of patients whose cells display PDN resistance in vitro. The clinical significance of high level of BCL-2 protein expression in hematological malignancies

TABLE I. Characteristics of B-Lineage ALL Patients

Pts	Sex/Age	FAB	Karyotype	Immunophenotype[a]	Result of induction therapy	Disease-free survival (mo)	Overall survival (mo)
1. SS[b]	F/14	L2	Normal	CD10+,CD34−,Cμ+	CR	25	28
2. AM[c]	M/44	L1–L2	Normal	CD10+,CD34+	CR	9	17+
3. CG[c]	M/39	L1–L2	t(8;14)	CD10+,CD34−	CR	30+	30+
4. FF[b]	M/50	L2	ND	CD10+,CD34+	CR	12+	12+
5. UG	M/19	L2	Normal	CD10+,CD34+,Cμ+	CR	2	9
6. MD[b]	F/34	L1–L2	t(9;22)	CD10+,CD34+,Cμ+	RES	—	5
7. BG[c]	M/29	L1–L2	t(9:22)	CD10+,CD34+,Cμ+	RES	—	3
8. SF[b]	M/33	L1–L2	Normal	CD10+,CD34+	CR	12	15
9. RR[c]	F/71	L3	ND	CD10+,CD34+	RES	—	3
10. VC	M/42	L1–L2	Normal	CD10−,CD34−	CR	43+	43+
11. CG[b]	F/57	L2	t(9;22)	CD10+,CD34+	RES	—	2
12. RM[b]	F/19	L3	t(4;11)	CD10−,CD34−	CR	15	17+
13. BM	M/35	L2	Hyperdyploid	CD10+,CD34+,Cμ+	CR	30	31+
14. GG	M/56	L2	t(2;22)	CD10+,CD34−	CR	28+	28+
15. PC	M/32	L1–L2	Normal	CD10+,CD34+	CR	17+	17+
16. PP[c]	F/30	L1	t(9;22)	CD10+,CD34+,Cμ+	CR	31	35+
17. ZE	M/15	L2	Hyperdyploid	CD10+,CD34+	CR	26+	26+
18. RA[c]	M/27	L2	ND	CD10+,CD34−	CR	11	36
19. VA[c]	F/61	L2	ND	CD10+,CD34+,Cμ+	CR	11	36
20. BB[b]	F/29	L2	Normal	CD10+,CD34−	CR	13	24
21. TG	M/31	L3	+2	CD10+,CD34+	RES	—	13

[a]In all cases, blast cells expressed CD19 and CD22 antigens.
[b]Patients whose cells showed less than 10% increase in BCL-2 protein production.
[c]Patients whose cells showed more than 25% increase in BCL-2 protein production.

TABLE II. Percentage of BCL-2 Positive Cells

Pts	Baseline	72 hr − no PDN	72 hr + PDN
1. SS	93.8	92.9	95.0
2. AM	87.9	88.2	88.6
3. CG	88.2	87.6	87.5
4. FF	88.1	87.8	87.6
5. UG	97.3	95.9	98.6
6. MD	98.9	99.3	99.1
7. BG	95.5	95.0	96.6
8. SF	92.8	93.6	92.0
9. RR	97.5	98.8	96.4
10. VC	90.3	91.0	88.1
11. CG	83.5	82.7	81.8
12. RM	91.2	93.2	93.3
13. BM	81.7	80.6	82.0
14. GG	88.4	89.2	88.6
15. PC	94.9	95.6	95.1
16. PP	85.3	84.7	86.8
17. ZE	92.5	93.7	93.7
18. RA	79.1	78.1	77.4
19. VA	86.3	84.3	88.8
20. BB	69.8	71.6	67.7
21. TG	85.9	88.7	84.4

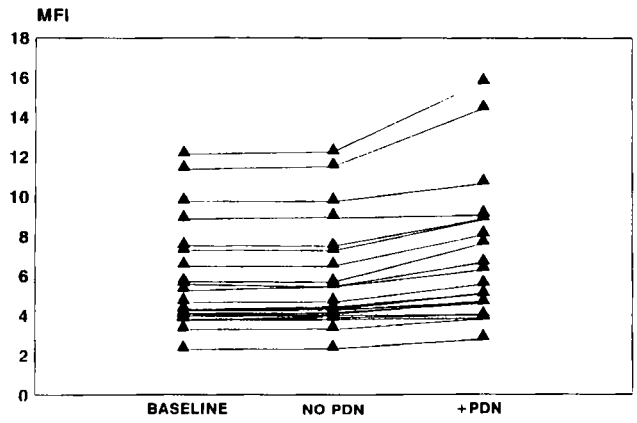

Fig. 2. Mean fluorescence index (sample mean channel : control mean channel ratio) of ALL cells from 21 patients after labeling with anti BCL-2 monoclonal antibody. The difference between untreated and PDN-treated samples is statistically significant.

is still controversial, as it has been reported that in childhood ALL the presence of BCL-2 is devoid of prognostic significance [9], at variance with adult AML, where cells showing a high BCL-2 level could represent a negative prognostic indicator [5,17].

In vitro studies have shown that overexpression of the *BCL-2* gene could confer resistance to apoptosis-inducing chemotherapeutic agents, including VP-16, methotrexate, doxorubicin, and PDN [8]. In particular, with gene transfer experiments, it has been demonstrated that introduction of the *BCL-2* gene into cell lines could reduce dexamethasone-induced apoptosis [7].

In this study, we have evaluated the expression of BCL-2 protein in B-lineage ALL cells from 21 patients upon in vitro treatment with a sublethal dose of PDN,

and we have demonstrated a significant increase in its expression in the majority of the cases. Similar results have been obtained by Tu et al. [11], by incubating a multiple myeloma cell line in the presence of VP-16 or Adriamycin. These findings need interpretation. It could be postulated that an increased BCL-2 protein production represents a possible mechanism of drug resistance by which drug-induced apoptosis is abolished. In order to support this hypothesis, it could be interesting to evaluate whether an increased BCL-2 protein production is observed in vivo as well, in adult ALL patient at relapse, and this could contribute to explain the poor cure rate observed in adult ALL [18]. According to our results, however, patients whose cells produced an increased amount of BCL-2 protein upon in vitro treatment with PDN did not show a poorer prognosis, but this could be because BCL-2 protein production was decreased or unchanged only in 6 out of 21 patients. In childhood ALL, it has been described that in vitro PDN resistance is increased at relapse, and this phenomenon appears to be non-p-glycoprotein mediated [13]. Another issue worth testing could be whether other compounds, either related or unrelated to the multiple drug resistance mechanism, could interfere with the level of BCL-2 protein production in blast cells. We cannot exclude, however, that induction of BCL-2 could be an epiphenomenon of other mechanisms of drug resistance. These data need to be confirmed in a larger series of samples and in vivo correlations should be performed.

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